HOXA10 is Expressed in Response to Sex Steroids at the Time of Implantation in the Human Endometrium

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Abstract

HOX genes are well-known transcriptional regulators that play an essential role in directing embryonic development. Mice that are homozygous for a targeted disruption of the Hoxa10 gene exhibit uterine factor infertility. We have recently demonstrated that HOXA10 is expressed in the adult human uterus. To examine expression of HOXA10 during the menstrual cycle, Northern blot analysis and in situ hybridization were performed. Expression of HOXA10 dramatically increased during the midsecretory phase of the menstrual cycle, corresponding to the time of implantation and increase in circulating progesterone. Expression of HOXA10 in cultured endometrial cells was stimulated by estrogen or progesterone. Stimulation of HOXA10 by progesterone was concentration-dependent within the physiologic range, and the effect of estrogen was inhibited by cycloheximide. These results identify sex steroids as novel regulators of HOX gene expression. HOXA10 may have an important function in regulating endometrial development during the menstrual cycle and in establishing conditions necessary for implantation in the human. (J. Clin. Invest. 1998. 101:1379–1384.) Key words: genes • homeobox • menstrual cycle • steroids • reproduction • pregnancy

Introduction

Uterine endometrium is one of the most dynamic tissues in the human body, undergoing dramatic developmental changes during each monthly menstrual cycle. Disorders of endometrial development lead to infertility, dysfunctional bleeding, endometriosis, and neoplasia. The molecular mechanisms that direct the ordered proliferation, differentiation, and cell death associated with the normal menstrual cycle are still poorly understood. Besides estrogen and progesterone receptors, no well-characterized transcription factors have been demonstrated to regulate gene expression in the developing endometrium. Hox genes are good candidates for regulating differentiation of the endometrium. Hox genes are transcriptional regulators that play essential roles in directing embryonic development (1). Hox genes are the vertebrate homologs of the Drosophila homeotic genes that determine the identity of specific body segments (1, 2). Recently, expression of Hox genes has been demonstrated in the developing reproductive system of the mouse (4, 5). Specifically, Hoxa10 has been shown to be expressed in the uterus of the fetal mouse (6). We have recently observed that expression of Hoxa cluster genes persists in the female reproductive tract, and that HOXA10 is expressed in the adult human endometrium (7).

In the mouse, Hoxa10 expression is essential for fertility. Mice carrying a targeted disruption of the Hoxa10 gene exhibit uterine factor infertility. Females ovulate normally, but do not support the preimplantation embryo or allow implantation. Mutant embryos are viable when placed in a surrogate (6). Taken together, these studies suggest that Hoxa10 is required for endometrial differentiation and for establishing the conditions required for implantation. We postulated that HOXA10 may have a similar function in the human. We observed that expression of HOXA10 in the human uterus is menstrual cycle stage–dependent, and identified sex steroids as novel regulators of Hox gene expression.

Methods

Tissue collection. Endometrium was collected from normal cycling women by endometrial biopsy under an approved Human Investigations Committee protocol. Half of the tissue was immediately frozen in liquid nitrogen and stored at −72°C. The other half of the tissue sample was fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Menstrual cycle dating was determined by menstrual history and confirmed by histological examination using the criteria of Noyes et al. (8).

Northern blot analysis. Tissues or cultured cells were homogenized in 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl, and 0.1 M 2-mercaptoethanol. Total RNA was size-fractionated on a 1% agarose/0.66 M formaldehyde gel, and transferred to nylon membranes. Membranes were hybridized with a 32P-labeled riboprobe as described below. Hybridization was performed overnight at 60°C in 50% formamide, 1× SSC, 5× Denhardt’s reagent, 0.2% tRNA, and 32P-labeled riboprobe at 2× 106 cpm/ml. The filter was washed twice at 68°C for 30 min in 0.1× SSC and 0.1% SDS, X-Omat AR film (Eastman Kodak Co., Rochester, NY) was exposed overnight at −70°C.

Probe preparation. Plasmids used for probe preparation were a generous gift from E. Boncinnelli, and have been well-characterized by this group and others (9, 10). pGEM plasmids containing 103 base pairs of the 3′ untranslated region of human HOXA10 were linearized with Eco RI or Hind III (New England Biolabs Inc., Beverly, MA), ethanol-precipitated, and used as template for generating riboprobes. Radiolabeled RNA probes were generated by in vitro transcription using the Riboprobe kit (Promega Corp., Madison, WI). Sense and antisense probes were generated using the appropriate

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RNA polymerase (T7 or SP6) and labeled with \([^{32}P]\) or \([^{33}P]\)UTP (Amersham, Arlington Heights, IL).

**In situ hybridization.** In situ hybridization was performed with both sense and antisense \(^{33}P\)-labeled riboprobes. Endometrium was fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, and then embedded in OCT compound (Miles Laboratories, Elkhart, IN). 10-µm frozen sections were obtained and mounted on Vectabond-coated slides (Vector Laboratories, Inc., Burlingame, CA). Before use, sections were treated with 0.2 M HCl, Pronase (0.16 mg/ml), and 0.026 M acetic anhydride, and were then dehydrated. Tissue sections were hybridized overnight with \(3\times10^{5} \text{ cpm} \) of each probe in 0.25 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.01 M NaPO4 (pH 6.8), 5 mM EDTA, Ficoll 400 (0.02%), polyvinylpyrrolidone (0.02%), BSA Fraction V (0.02%), 50% formamide, 12.5% dextran sulfate, yeast tRNA (1.25 mg/ml), and 10 mM DTT. Hybridization was performed in a humidified chamber for 16 h at 50°C. Slides were treated with RNase A at 37°C, and were then washed for 16 h in 0.25 M NaCl, 0.01 M Tris-Cl (pH 7.5), 0.01 M sodium phosphate (pH 6.8), 5 mM EDTA, Ficoll 400 (0.02%), polyvinylpyrrolidone (0.02%), BSA Fraction V (0.02%), and 50% formamide. Slides were dehydrated, dried, and dipped in K5D (Ilford Limited, Mobberley, Cheshire, United Kingdom) emulsion. Exposure was carried out at 4°C for 7–12 d, and slides were developed with Kodak D-19 (Eastman Kodak Co.). Slides were counterstained with hematoxylin and eosin. Representative darkfield and bright-field photomicrographs were taken at 20× on a microscope (Olympus Corp., Lake Success, NY) with Kodak Ektachrome film (Eastman Kodak Co.).

**Cell culture.** Endometrial samples were obtained from four different normal cycling women in the proliferative phase. Endometrial epithelium and stromal cells were separated as described previously (11). In brief, the tissue was finely minced, and cells were dispersed by incubation in HBSS containing Hapes (25 mM), penicillin (200 U/ml), streptomycin (200 µg/ml), collagenase (1 mg/ml, 15 U/mg), and DNase (0.1 mg/ml, 1,500 U/mg) for 20–30 min at 37°C with agitation. The cells were separated by filtration through a wire sieve with 73-µm diameter pores. The stromal cells are found in the filtrate, whereas the endometrial glands are retained by the sieved. The stromal cells were pelleted, washed, and suspended in phenol red–free Ham’s F12/DME (1:1) containing charcoal-stripped FCS. The cells were passaged once and grown to confluence. Confluent monolayers were maintained in serum-free media for 48 h, and subsequently treated with 17 β estradiol (5 × 10^{-8} M) or medroxyprogesterone acetate (1 × 10^{-7} M). Immunocytochemical analysis of endometrial cells was conducted after the first passage. Factor VIII, cytokeratin, 3C10, and vimentin were used as markers of endothelial cells, epithelial cells, macrophages and stromal cells, respectively. 97% of the cells were endometrial stromal cells. Epithelial cells and macrophages accounted for 97% of the cells; epithelial cells, macrophages and stromal cells, respectively. 3% and 0.2% of the cells; epithelial cells were absent. Estrogen and progesterone receptor status was verified by ELISA according to the manufacturer’s instructions (Abbot Laboratories, Weisbaden, Germany). Ishikawa cells were grown in the same medium and treated identically to the primary stromal cells.

**Statistical analysis.** The autoradiographic bands were quantified using a laser densitometer (Molecular Dynamics, Sunnyvale, CA). Each HOXA10 band was normalized to the value obtained from the same lane hybridized to G3PDH. Data were analyzed using ANOVA. Statistical significance was defined as \(P < 0.05\).

**Results**

**Endometrial HOXA10 expression is menstrual cycle stage-dependent.** To examine the role of HOXA10 in the cyclic development of the endometrium, the menstrual cycle stage–specific expression pattern was determined. Total RNA was extracted from human endometrium collected from normal cycling women. The 30 specimens were divided into approximately equal groups corresponding to early and late proliferative stage, and to early, mid, and late secretory stage endometrium. An additional sample of pregnancy decidua was also examined. Northern blot analysis was performed using an antisense \(^{32}P\)-labeled riboprobe specific to the 3’ untranslated region of HOXA10. Representative results are shown in Fig. 1. a. Expression is evident throughout the menstrual cycle, but increases in the midsecretory phase. Hybridization to a control
Hormone agonist, in comparison with levels of medroxyprogesterone acetate or gonadotropin-releasing hormone, is significantly lower in the endometrium of patients treated with long-term medication. Chronic anovulation shows low secretory endometrium. Additionally, endometrium from patients with pharmacologically modified or pathologic states is altered in nonreceptive endometrium. Northern analysis was performed on endometrial samples from three women in the late secretory phase of pregnancy as demonstrated in Fig. 2a. Northern blot analysis of endometrium spanning the menstrual cycle, and markedly increases at the mid and late secretory phase, confirming the results of the Northern blot analysis (not shown).

HOXA10 expression in endometrium from the late secretory phase of the menstrual cycle (S) and the decidualized endometrium of pregnancy (D). A representative autoradiogram is shown after exposure for 24 h. HOXA10 expression persists into pregnancy. Expression of HOXA10 is altered in nonreceptive endometrium. Northern analysis was performed on endometrial samples from three women in the late secretory phase (S) and three women with each of the following conditions: long-term treatment with medroxyprogesterone acetate (MPA), treatment with gonadotropin-releasing hormone analog (GnRH), and chronic anovulation (AnOv). Densitometric analysis normalized to G3PDH is shown. Error bars are SEM. *Statistically different from secretory endometrium.

HOXA10 expression in proliferative endometrium as demonstrated by in situ hybridization. HOXA10 is expressed in both glandular and stromal cells. Levels of expression appeared higher in the mid and late secretory phase, confirming the results of the Northern analysis (not shown).

HOXA10 expression is modulated by sex steroids in endometrial cell culture. The increased HOXA10 expression observed in the midsecretory endometrium coincided with the time of increased circulating progesterone. To test whether HOXA10 expression is regulated by sex steroids, HOXA10 expression was measured in cultured cells after treatment with estrogen and progesterone. Endometrial samples from the proliferative phase of the menstrual cycle were used as a source of primary cultures of stromal cells. Cells were grown to confluence in steroid-free media, and were serum-starved before treatment with physiologic concentrations of 17-beta estradiol, medroxyprogesterone acetate, or both. RNA was extracted and subjected to Northern blot analysis. Fig. 4a shows that estrogen stimulated HOXA10 expression approximately twofold compared with control cells that were not exposed to steroids. Treatment with progesterone stimulated HOXA10 expression to a greater degree than did estrogen alone. Combined treatment with estrogen and progesterone was additive. Average densitometry readings are displayed in Fig. 4b, confirming the response to sex steroids in independent cell lines. These cells show similar HOXA10 expression after exposure to concentrations of estrogen beyond physiologic ranges (10^{-10}-10^{-6} M). A dose responsive expression over the physiologic range of progesterone concentration is displayed in Fig. 5. These findings are consistent with the observed increased expression of HOXA10 in the endometrium of the secretoryormemum. Expression is noted in both glandular and stromal cells. Fig. 3c shows a high-power brightfield view of HOXA10 expression in proliferative endometrium as demonstrated by in situ hybridization. HOXA10 is expressed in both glandular and stromal cells. Levels of expression appeared higher in the mid and late secretory phase, confirming the results of the Northern blot analysis (not shown).

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Figure 2. (a) Northern blot analysis of HOXA10 expression in endometrium from the late secretory phase of the menstrual cycle (S) and the decidualized endometrium of pregnancy (D). A representative autoradiogram is shown after exposure for 24 h. HOXA10 expression persists into pregnancy. Expression of HOXA10 is altered in nonreceptive endometrium. Northern analysis was performed on endometrial samples from three women in the late secretory phase (S) and three women with each of the following conditions: long-term treatment with medroxyprogesterone acetate (MPA), treatment with gonadotropin-releasing hormone analog (GnRH), and chronic anovulation (AnOv). Densitometric analysis normalized to G3PDH shows that approximately equal amounts of RNA were loaded into each lane. Densitometric analysis was performed on each sample, and Fig. 1b shows the average abundance of HOXA10 during each stage of the menstrual cycle normalized to G3PDH. There was no statistical difference between the levels of expression in any of the segments of the proliferative phase or the early secretory phase. In contrast, expression at the mid and late secretory phase was significantly elevated relative to earlier stages. Expression in the mid and late secretory phase was not significantly different. Taken together, these results indicate that HOXA10 expression varies during the menstrual cycle, and markedly increases at the mid-secretory phase, which corresponds to the time of implantation. Additionally, HOXA10 expression persists in the decidua of pregnancy as demonstrated in Fig. 2a.

HOXA10 is expressed at low levels in the endometrium of women with pharmacologically modified or pathologic states of altered endometrial development that lack receptivity to implantation. Fig. 2b shows that HOXA10 levels are significantly lower in the endometrium of patients treated with long-term medroxyprogesterone acetate or gonadotropin-releasing hormone agonist, in comparison with levels of medroxyprogesterone acetate or gonadotropin-releasing hormone acetate (MPA), treatment with gonadotropin-releasing hormone analog (GnRH), and chronic anovulation (AnOv). These findings are consistent with the observed increased expression of HOXA10 in the endometrium of the secretory
phase of the menstrual cycle when estrogen is present and progesterone is increased.

To verify that sex steroids stimulate HOXA10 expression, studies were performed using Ishikawa cells, a well-differentiated endometrial adenocarcinoma cell line derived from the glandular component of the endometrium (12, 13). The presence of estrogen and progesterone receptor was documented by ELISA (not shown). Ishikawa cells were cultured under similar conditions as stromal cells, and treated with the same concentrations of sex steroids. Fig. 6 shows that Ishikawa cells express HOXA10, and that expression increases approximately twofold after treatment with either estrogen or progesterone. In contrast to stromal cells, the effects of estrogen and progesterone are not additive. To assure that the progesterone effect on HOXA10 expression is mediated through the progesterone receptor in Ishikawa cells, the cells were pretreated with the antiprogestrone RU486. RU486 blocked the response to progesterone. These results confirm a regulatory role for sex steroids on HOXA10 expression. In Ishikawa cells, maximal levels of HOXA10 mRNA were reached within 1 h of exposure to estrogen or progesterone, and remained elevated at 8 h (not shown). Treatment with cycloheximide before adding steroid hormones had no effect on the increase in HOXA10 expression, suggesting that new protein synthesis was not required (Fig. 7).

**Discussion**

One way in which differential tissue identity is obtained is through selective activation of HOX genes. The role of HOX genes in differentiation of embryonic tissues is well-established. We have recently described the role of HOX genes of the A axis in mullerian system development, and their persistent expression in the adult female reproductive system (7). HOXA10 is expressed in the glands and stroma of the adult endometrium.

Endometrial glands and stroma undergo cyclic developmental changes in response to circulating sex steroids. We
demonstrate that *HOXA10* is expressed in the endometrium in a menstrual cycle stage-dependent fashion. *HOXA10* expression is noted in the proliferative phase of the menstrual cycle when estrogen is the predominant steroid hormone affecting the uterus. *HOXA10* mRNA levels dramatically increase in the midsecretory phase at the time when progesterone levels rise rapidly. A parallel increase in expression is noted in cell culture experiments in which both primary stromal cells and Ishikawa endometrial adenocarcinoma cells increase *HOXA10* expression in response to estrogen and progesterone. It is possible that differential *HOXA10* expression in response to changing estrogen and progesterone concentrations leads to sequential differentiation of the endometrium. *HOX* genes may play a role in the menstrual cycle, altering cell fate during differentiation of the endometrium.

During vertebrate development, morphogens convey positional information and selectively alter the expression of essential regulatory genes. Previously, *HOX* genes have been shown to be regulated by the morphogen retinoic acid (14, 15). The retinoic acid receptor belongs to the family of nuclear hormone receptors that includes the estrogen and progesterone receptors. Here we show that *HOXA10* expression is modulated by other nuclear hormone receptor ligands, estrogen, and progesterone, presumably acting through their respective receptors. Growth and differentiation of the uterine endo-

![Figure 4](image1.png)

**Figure 4.** (a) Northern blot analysis of *HOXA10* expression in cultured primary endometrial stromal cells. RNA from control cells (C) that were not exposed to steroid shows basal levels of *HOXA10* expression. Cells were treated with $5 \times 10^{-8}$ M estrogen (E), $1 \times 10^{-7}$ M progesterone (P), or both hormones for 4 h. *HOXA10* expression increased with estrogen or progesterone. The effects of estrogen and progesterone were additive. A representative autoradiogram is shown. (b) Densitometric analysis of *HOXA10* expression from four stromal cell cultures obtained from different patients. Data were normalized to G3PDH. A statistically significant difference was observed between each lane. Error bars are SEM.

![Figure 5](image2.png)

**Figure 5.** Dose response of *HOXA10* expression after treatment with progesterone. Primary stromal cells were treated with varying concentrations of progesterone, and Northern blot analysis was performed. *HOXA10* expression relative to G3PDH control is shown. An increase in *HOXA10* expression was noted over the physiologic range.

![Figure 6](image3.png)

**Figure 6.** Expression of *HOXA10* in Ishikawa cells in response to sex steroids. Cells that were demonstrated to be estrogen- and progesterone receptor–positive showed *HOXA10* expression in the untreated state (C). Treatment with $5 \times 10^{-8}$ M estrogen (E), $1 \times 10^{-7}$ M progesterone (P), or both hormones (E+P) resulted in similar stimulation of *HOXA10* expression. The progesterone-induced expression is blocked by $1 \times 10^{-6}$ M RU486 (P+RU). Average densitometry results normalized to G3PDH are shown. Error bars are SEM. *Statistically different from control.
metrium during the menstrual cycle and pregnancy is governed principally by progressive changes in the levels of sex steroids. As HOX genes respond to the morphogen retinoic acid during embryonic development, they may respond to sex steroids in the developing adult uterine endometrium.

Estrogen and progesterone are novel regulators of HOX gene expression. Besides retinoic acid, few regulatory molecules inducing HOX expression are known. The rapid induction of HOX10 expression in response to sex steroids as well as the lack of inhibition by cycloheximide suggest that steroid hormones and their receptors likely bind directly to regulatory elements of HOX10. This is the first demonstration of a steroid hormone acting as a direct regulator of HOX expression. It will be interesting to determine if other HOX genes respond to these steroids.

Targeted disruption of the Hoxa10 gene in the mouse results in an infertility phenotype (6). Females lacking Hoxa10 have a uterine factor defect that results in death of the preimplantation embryo and failure of implantation. Homozygous mutants ovulate normally, and produce oocytes that can be fertilized with sperm from a wild-type male. When transferred to a surrogate wild-type uterus, these embryos develop normally, but wild-type embryos fail to implant in the Hoxa10 mutant uterus.

The location and timing of expression in the uterus is consistent with HOX10 playing a similar role in humans. HOXA10 is differentially expressed in the uterine endometrium during the menstrual cycle. A dramatic increase in levels of expression accompanies endometrial differentiation at the midsecretory phase, which is the time of implantation in the human. A striking conservation of gene function between species is commonly observed (16). The homeobox of the murine Hoxa10 gene is 95% conserved with the human gene at the nucleotide level, and 100% conserved at the amino acid level (17). Most genes that are known to be expressed in the secretory phase endometrium do not have a necessary role in implantation; targeted mutations have typically failed to demonstrate an effect on the ability of the uterus to support an embryo. In contrast, Hoxa10 is essential for implantation in the mouse. Functional conservation between species implies that these two genes (human HOXA10 and murine Hoxa10) with highly conserved sequences have a similar role in both species. The timing and location of expression in the human secretory endometrium further supports an essential function in human implantation. Furthermore, the persistent expression in decidua suggests a continued role in pregnancy maintenance.

Acknowledgments

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References